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Gene Expression Profile in *Neisseria meningitidis* and *Neisseria lactamica* upon Host-Cell Contact

From Basic Research to Vaccine Development

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ABSTRACT

Differential gene regulation in the human pathogen *Neisseria meningitidis* group B (MenB) and in *Neisseria lactamica*, a human commensal species, was studied by whole genome microarray after bacterial interaction with epithelial cells. Host-cell contact induced changes in the expression of 347 and 285 genes in MenB and *N. lactamica*, respectively. Of these, only 167 were common to MenB and *N. lactamica*, suggesting that a different subset of genes is activated by pathogens and commensals. Change in gene expression was stable over time in *N. lactamica*, but short-lived in MenB. A large part (greater than 30%) of the regulated genes encoded proteins with unknown function. Among the known genes, those coding for pili, capsule, protein synthesis, nucleotide synthesis, cell wall metabolism, ATP synthesis, and protein folding were down-regulated in MenB..Transporters for iron, chloride and sulfate, some known virulence factors, GAPDH and the entire pathway of selenocysteine biosynthesis were upregulated. Gene expression profiling indicates that approximately 40% of the regulated genes encode putative surface-associated proteins, suggesting that upon cell contact *Neisseria* undergoes substantial surface remodeling. This was confirmed by FACS analysis of adhering bacteria using mouse sera against a subset of recombinant proteins. Finally, a few surface-located, adhesion-activated antigens were capable of inducing bactericidal antibodies, indicating that microarray technology can be exploited for the identification of new vaccine candidates.

Key Words: gene regulation • *Neisseria meningitidis* • *Neisseria lactamica* • vaccine development • microarray

Abbreviations: MenB-*Neisseria meningitidis* group B • STM-signature tagged mutagenesis • D-MEM-Dulbecco's Modified Eagle l Medium • GAPDH-glyceraldehyde 3-phosphate dehydrogenase • ORF-open reading frame • FCS-fetal calf serum

INTRODUCTION

Neisseria meningitidis and *Neisseria lactamica* are related gram-negative bacteria, which colonize the human nasopharynx, but differ in the final outcome. *N. lactamica* is a commensal, which never causes disease. Conversely, *N. meningitidis* eventually spreads from the nasopharynx to the bloodstream and the meninges causing severe meningitis and sepsis in children and young people. The disease is fatal in 5 to 15% of cases and causes severe neurological sequelae in up to 25% of survivors.

Although enormous progress has been made over the last several years, our understanding of meningococcal invasion and infection is still incomplete (for recent reviews see [refs. 1 and 2]). New genomic technologies and the recent elucidation of *N. meningitidis* group B (MenB) genome sequence[3] are now expected to facilitate the study of *Neisseria* interaction with its human host.

In this study, DNA microarrays carrying the entire gene repertoire of the MC58 strain have been exploited to analyze changes in gene expression induced in *N. lactamica* and MenB upon interaction with human 16HBE14 epithelial cells. With this kind of analysis, three interesting pieces of information were obtained. First of all, the comparison of the gene activation profiles in MenB and *N. lactamica* led to the identification of genes regulated in both organisms and genes, which are specific for MenB. This latter set of genes is expected to play an important role in MenB virulence and pathogenicity. Secondly, several regulated genes (approximately 40%) encoded peripherally located proteins, indicating that host interaction induces a profound remodeling of the bacterial cell membrane. This observation was confirmed by FACS analysis of MenB cells using antibodies against selected recombinant surface proteins. Finally, when 12 upregulated proteins were tested for their capacity to induce bactericidal antibodies, five of them were positive in this assay indicating that DNA microarray technology can represent a valid approach to new vaccine discovery.

DNA microarrays have been used recently to study the gene expression profile in eukaryotic cells infected with different pathogens,[4-7] however studies focusing on the bacterial genes have not been published so far.[8] This work not only provides an overall picture of the transcriptional changes induced in bacteria upon host interaction, but also reveals novel aspects of meningococcal pathogenicity and paves the way to important applications of this technology.

MATERIALS AND METHODS

Bacterial Strains and Cell Cultures

MenB MC58 and *N. lactamica* NL19 were grown on GCB agar (BD Biosciences, Franklin Lakes, NJ) supplemented with 4g/L glucose, 0.1g/L glutamine, 2.2mg/L cocarboxylase at 37°C in 5% CO_2 for 16 hours. Adhesion assays were performed on 16HBE14, a polarized human bronchial epithelial cell line transformed with SV40

large T-antigen, kindly provided by R. Moxon (Oxford University, UK). Cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum (FCS), 1.5mM glutamine and 100μg/mL kanamycin sulfate.

Adhesion Experiments

Bacteria colonies from 16-hour old plates were suspended in D-MEM medium at a final OD_{600} value of 1 and 0.4mL of suspension (about 10^9 bacteria) were added to epithelial cells ($2x10^6$) and incubated at 37°C in 5% CO_2 at different times. Cell-adhering bacteria were colony counted after extensive washing (four times) of epithelial cells with 5mL Hanks' Balanced Salt Solution (HBSS)-2% FCS (Life Technologies, Paisley, Scotland), followed by cell lysis with 1% saponin in HBSS for 10 min at 37°C. Nonspecific binding of bacteria to plastic was estimated following the same procedure described above in the absence of epithelial cells.

Microarray Preparation

DNA microarrays were prepared using DNA fragments of each annotated open reading frame (ORF) in the MenB MC58 genome, as published by Tettelin *et al.*[3] (http://www.tigr.org). PCR primers were selected from a MULTIFASTA file of the genomic ORFs using either Primer 3 (http://www.genome.wi.mit.edu/genomesoftware/other/primer3.html) or Primer Premier (Premier Biosoft, Ca, USA) software, and the support of locally developed "PERL" scripts for handling multiple nucleotide sequence sets. The majority of PCR primer pairs were 17-25 nucleotides long and were selected within the ORF sequences so as to have an average annealing temperature of around 55°C (range 50 to 60°C) and produce amplified products of 250-1,000bp (when possible a length of 600-800 bp was selected). For ORFs shorter than 250bp, primers annealing as close as possible to the start and stop codons were selected. In total, 2,121 out of 2,158 genes were amplified. Considering that the nonamplified genes correspond to duplicated sequences, 100% of the identified ORFs were represented on the chips. Amplification reactions were performed on MC58 genomic DNA with a Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA), and using Taq polymerase (ROCHE Diagnostic, Mannheim, Germany) as recommended by the manufacturer. PCR products were purified using Qia-Quick spin columns (Qiagen, Chatsworth, CA) and quantified spectrophotometrically at OD_{260}.

Array printing was performed using a Gen III spotter (Amersham Biosciences, Uppsala, Sweden) on type VII aluminum coated slides (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. Thirty-seven different eukaryotic and prokaryotic genes were included in the chips as positive and negative controls. To establish the stringency of hybridization conditions, six sequences in the 73-100% homology range to a spiked control RNA were also included as controls. Hybridization conditions were set to detect hybridization signals of sequences having at least 73% homology.

Microarray Hybridization and Analysis

Microarray analysis was carried out comparing the profile of total RNA extracted from bacteria growing in D-MEM-10% FCS culture medium and bacteria adhering to epithelial cells. Cell-adhering bacteria were prepared as described above. Total RNA was extracted from bacterial pellets using RNeasy spin columns (Qiagen, Chatsworth, CA). Bacterial RNA was quantified by one-step quantitative RT PCR of the 16S rRNA using LightCycler equipment (ROCHE Diagnostic, Mannheim, Germany). For RNA labeling, 1.5μg was reverse transcribed using Superscript II™ reverse transcriptase (Life Technologies, Paisley, Scotland), random 9-mer primers and the fluorochromes Cy-3 dCTP and Cy-5 dCTP (Amersham Biosciences, Uppsala, Sweden). Cy-3 and Cy-5 labeled cDNAs were copurified on Qia-Quick spin columns (Qiagen, Chatsworth, CA). The hybridization probe was constituted by a mixture of the differently labeled cDNAs derived by cell-adhering bacteria and bacteria growing in liquid medium. Probe hybridization and

washing were performed as recommended by the slide supplier (Amersham Biosciences, Uppsala, Sweden). Slides were scanned with a GIII scanner (Amersham Biosciences, Uppsala, Sweden) at 10μm per pixel resolution. In each experiment the two RNA samples were labeled in the direct (Cy3-Cy5) and reverse (Cy5-Cy3) labeling reaction to correct for dye-dependent variation of labeling efficiency. The resulting 16-bit images were processed using the Autogene program (version 2.5, BioDiscovery, Inc., Los Angeles, CA). For each image, the signal value of each spot was determined by subtracting the mean pixel intensity of the background value, and normalizing to the median of all spot signals. The spots, which gave a negative value after background subtraction, were arbitrarily assigned the standard deviation value of negative controls. The data resulting from direct and reverse labeling were averaged for each spot. Expression ratios were obtained at each timepoint dividing hybridization signals from adhering bacterial RNA by nonadhering bacterial RNA. The data from each timepoint represents the average of at least four independent experiments. Genes whose expression ratios changed by at least twofold (P-values < 0.01) were considered up- or down-regulated. Expression pattern analysis and data visualization were done using Gene-Spring software (version 3.1.0, Silicon Genetics, Redwood City, CA).

Protein Expression and Immunization Procedures

Genes were PCR-amplified from MC58 chromosomal DNA by using 30-nucleotide-long specific primers annealing at the 5' and 3' ends of each gene. In all cases, the leader sequence for secretion was replaced with the ATG codon to drive the expression of the recombinant proteins in the cytoplasm of *E. coli*. Cloning and purification were performed as already described.[9] Briefly, proteins were expressed as His-tagged and GST fusions. His-tagged recombinant proteins were purified by metal ion affinity chromatography (IMAC), whereas glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) was used for GST-fusion purification. 20μg of each purified protein was mixed with Freund's adjuvant and used to immunize CD1 mice at days 1, 21 and 35. Blood samples were taken on day 34 and 49.

For Western blot analysis, total bacterial extracts were separated on 12-15% polyacrylamide-SDS gels and proteins were detected with 1:100 mouse sera dilutions.

FACS Analysis

Adhering bacteria were collected after saponin treatment, washed with PBS-1% BSA and centrifuged. The bacterial capsule was permeabilized by dropwise addition of cold 70% EtOH directly on the pellet at -20°C for one hour. Bacteria were washed, resuspended with PBS-1% BSA at the desired density and incubated either with sera from mice immunized with meningococcal recombinant proteins or with pre-immune sera[9] for two hours on ice. After washing, bacteria were subsequently incubated with R-phycoerythrin-conjugated goat $F(ab)_2$ anti-mouse IgG (Cedar Lane Laboratories, Hornby, Canada) for 1 hour on ice to detect antibody binding. Bacteria were washed and finally fixed with 0.25% *para*-formaldehyde and analyzed for cell-bound fluorescence using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Negative controls included noninfected human 16HBE14 epithelial cells subjected to the procedures described above.

Bactericidal Assay

Bactericidal activity of immune sera was tested against strains MC58, 2996 and mutants using pooled baby rabbit serum (Cedar Lane Laboratories, Hornby, Canada) as complement source, as described.[9] Essentially, bacteria were grown in Mueller-Hinton broth containing 0.25% Glucose, to OD620 of 0.23, and diluted to 105 CFU/mL in assay buffer (50 mM phosphate buffer, 10 mM MgCl2, 10 mM CaCl2, and 0.5% BSA, ph7.2). The serum bactericidal activity assay was performed using 25mL of test serum dilutions, 12.5μL of diluted bacteria and 12.5μL of baby rabbit complement. Controls included bacteria incubated with preimmune serum and with heat-inactivated serum

complement. For colony counting, 10μL bacteria were plated on Mueller-Hinton agar plates at time 0 and after one-hour incubation at 37°C. Bactericidal titers were given as the reciprocal of the serum dilution yielding at least 50% bacterial killing.

RESULTS

Kinetics of Adhesion of *Neisseria* Strains to Epithelial Cells

Adhesion to the epithelial cells of the nasopharyngeal tract represents the first step of *Neisseria* infection. To study the kinetics of bacterial adhesion and find the optimal conditions for microarray experiments, *N. meningitis* and *N. lactamica* were incubated with the 16HBE14 human cell line. Samples were taken at time 0 and after 30, 60, 120, and 180 minutes of cocultivation. As shown in Figure 1, adhesion kinetics were similar for the two bacteria. After one hour of cocultivation, approximately 5-10 bacteria were found associated to each cell. This number increased with time, to reach 70-150 bacteria/cell after three hours, and paralleled the growth rate of MC58 in D-MEM culture medium. A large part of the time-dependent increase in cell-associated bacteria was due to new adhesion events taking place between cells and bacteria freely growing in the medium. In fact, when bacteria were incubated with the cells for one hour and the nonadhering bacteria were removed, the proliferation of cell-associated bacteria was negligible.

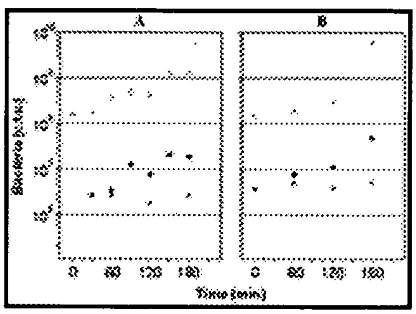

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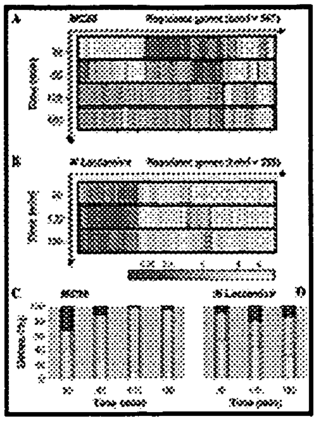
FIGURE 1. Adhesion and growth kinetics of MenB **(A)** and *N. lactamica* **(B)**. Bacterial growth in D-MEM-10% FCS medium (▲) was determined by plating aliquots of the culture at different times. To evaluate the growth rate of cell-adhering bacteria, both strains were incubated with HBE14 epithelial cells for 1 hour and nonadhering bacteria were removed by extensive washing. Fresh sterile medium was added and adhering bacteria were counted at different times after lysis of epithelial cells (●). Finally, the kinetics of bacterial association was determined by adding bacteria to epithelial cells and cell-adhering bacteria were counted at different times after cell lysis (■).

Panoramic View of Cell Contact-Induced Changes in Gene Expression

To study the changes in gene expression induced by host cell contact, RNA was purified from cell-adhering bacteria and bacteria grown in 16HBE14 culture medium. Figure 2 is a color-coded representation of the whole microarray analysis of MenB and *N. lactamica* during interaction with 16HBE14 epithelial cells. Overall, 347 and 285 genes altered their expression in MenB and in *N. lactamica*, respectively.

FIGURE 2. Kinetics of the gene expression pattern in MenB **(A)** and *N. lactamica* **(B)** adhering to epithelial cells. Bacteria (10^9) were added to 16HBE14 epithelial cells (2×10^6) and RNA was prepared at different times from adhering and freely growing bacteria. *Top panels* show clustered expression profiles of genes whose regulation differs from freely growing bacteria by at least twofold at any timepoint.



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Bottom panels group the same regulated genes as in the top panels according to their activation state (upregulated genes, *light gray*; downregulated genes, *dark gray*) to give a visual indication of the persistence of gene regulation.

Only 167 of the regulated genes were common to both bacteria, indicating that while a common set of genes responds to cell-contact, the different behavior of the two bacteria most likely resides in the 180 and 118 genes specifically regulated in MenB and *N. lactamica*, respectively.

A relevant difference between MenB and *N. lactamica* is the time of persistence of RNA species in a cell-adhering population. As clearly evident from the comparison of Figure 2A and B, while the number of regulated RNA species markedly decreased with time in MenB, 30% of the adhesion-specific *N. lactamica* RNAs remained regulated throughout the analysis and most of the regulated genes remained either in the activation or in the downregulation state for a longer period of time.

The difference in mRNA levels between the two strains may be a consequence of different mechanisms of transcription regulation and/or RNA stability. It is interesting to note that six transcription regulators were found regulated during adhesion in MenB as opposed to the three regulators (NMB1561, NMB1511 and crgA (NMB1856)) regulated in *N. lactamica*. Furthermore, STM analysis by Sun *et al.*[10] has shown that inactivation of the RNAse genes NMB0686 and NMB0758 conferred an attenuated phenotype to MenB, suggesting the need for rapid RNA turnover.

While the biological significance of the difference in RNA persistence between MenB and *N. lactamica* remains to be thoroughly investigated, it is tempting to speculate that the phenomenon is linked to the different relationship the two bacteria have with the human host. *N. lactamica* has evolved to become commensal and nasopharyngeal epithelium represents its final destination. Therefore one would expect that once the bacterium comes into contact with epithelial cells the program of RNA and protein synthesis would remain more or less unaffected until substantial environmental variations occur. In contrast, MenB has the potential of moving from the epithelium to the endothelium and eventually of invading the blood stream and the meninges. This implies a transient interaction with epithelial cells and a propensity to reorganize its transcription and translation profiles to rapidly adapt itself to new environmental situations.

A Close View of Cell Contact-Induced Changes in Gene Expression

Cell Contact Induces Reduced Metabolism

An interesting observation derived from the microarray analysis of the transcriptional events occurring after cell contact is that, in agreement with the growth reduction curve shown in Figure 1, both *N. meningitidis* and *N. lactamica* reduce the activity of many growth-dependent genes. The list of downregulated genes in MenB includes 34 genes involved in

protein synthesis, five genes implicated in nucleotide synthesis, and seven genes of cell wall septation and synthesis. Reduction of transcription activity also involved the gene cluster encoding the ATP synthase F1 and F0 subunits (atpC (NMB1933), atpD (NMB1934), atpG (NMB1935), atpA (NMB1936), atpH (NMB1937), atpF (NMB1938), and atpB (NMB1940)). This can be explained by an overall lower demand for ATP due to the reduced bacterial growth once associated to the cells. Alternatively, it is tempting to speculate that, once cell associated, bacteria are able to utilize part of the ATP synthesized by the host. Many of these metabolic genes (27 genes) were also downregulated in *N. lactamica*, indicating that in both species the interaction with epithelial cells is at least partially mediated by similar events and a reduced metabolic demand.

Upregulation of Transporters

A second common event occurring in the two species appears to be the activation of some transport systems involved in transmembrane trafficking of different compounds. Commonly upregulated transport machineries include the amino acid tranporter gene NMB0177, the ABC transporters NMB0098 and NMB0041, the sulfate transporter gene cysT (NMB0881) and the ABC Fe^{3+} transporter gene NMB1990. Activation of genes involved in iron transport is intriguing since our experimental conditions were not iron limiting. Considering that, together with the ABC transporter gene, the transferrin binding protein gene (tbp1 (NMB0461)) and the oxygen-independent coprophorphyrinogen III oxydase gene (NMB0665) were also activated in both species, the data would suggest that of the three possible iron acquisition pathways,[11] adhering bacteria preferentially take up iron from transferrin.

Activation of transmembrane trafficking appears to be more pronounced in MenB. In fact, other transporter genes were specifically regulated in this organism and include the ABC cassette constituted by the three genes NMB0787, NMB0788, NMB0789, the amtB (NMB0615) transporter for ammonium, the ABC sulfate transporter (cysA (NMB0879), cysW (NMB0880), cysT (NMB0881), sbp (NMB1017)), the iron ABC transporter fbpA (NMB0634), the efflux pump gene NMB1719 and the chloride transporter gene NMB2006. It is interesting to note that NMB2006 is one of the 73 genes whose inactivation conferred an attenuated phenotype to MenB.[10] Furthermore, activation of the sulfate transport system, which is strictly linked to sulfur-containing amino acid metabolism, is probably the most evident difference between cell-adhering MenB and *N. lactamica* (see below).

Adhesion

In studying the biology of MenB invasion, a large number of experimental data have shown that after a first phase of localized adherence in which pili play an essential role, the genes of pili biosynthesis are downregulated to allow intimate attachment and diffuse adherence.[12] Our data show that the pilE gene (NMB0018), whose product contributes to the interaction with epithelial cells and the induction of cortical plaques, was slightly upregulated after 30 minutes. Furthermore, the pilC (NMB1847) transcript, encoding the major pilus adhesin involved in initial attachment to cells, was also marginally present in cell-associated bacteria after 30 minutes. However, at 30-minute incubation, crgA /NMB1856), the negative regulator of pilC1 expression,[13] was already clearly upregulated. In addition, pilT (NMB0052) RNA, whose product is responsible for pili retraction,[14] although not upregulated, was one of the most abundant RNA species among total bacterial RNAs (data not shown). As for the other pili genes, they appeared poorly transcribed and pilP (NMB1811) was downregulated.

Intimate attachment requires the involvement of membrane-associated proteins interacting with specific cellular receptors. Several bacterial proteins have been proposed, the best candidates being the Opa/Opc proteins, porins, and adhesins. Our microarray data on MenB show that the opa/opc genes and the porin genes were not regulated during adhesion but were very actively transcribed throughout the three-hour incubation (data not shown). Furthermore, MafA adhesins (mafA-1 (NMB0375), mafA-2 (NMB0652)) were upregulated at the beginning of our kinetics analysis and the macrophage infectivity potentiator (MIP)-related protein (NMB0995) was constantly upregulated. The expression of

MIP genes is characteristic of intracellular pathogens and is known to increase their survival inside infected host cells.[15-17]

When expression of adhesion genes was analyzed in *N. lactamica*, a similar transcriptional pattern was observed, with the exception of mafA-1 that is MenB specific. Therefore, apart from mafA-1 and a few additional pilin genes specific for *N. lactamica* (data not shown), the overall expression profile would indicate that the two bacterial species utilize similar mechanisms of adhesion to epithelial cells.

Upregulation of Amino Acids and Selenocysteine Biosynthesis

IVET and STM technologies have shown that amino acid metabolism plays an important role in the infective process of many pathogens, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Salmonella typhimurium*, and *Brucella suis*.[18] In agreement with these observations, our microarray analysis indicates that 16HBE14-associated MenB and *N. lactamica* upregulated some of the genes involved in the synthesis of several amino acids. In MenB, a more pronounced activation involves histidine, methionine, cysteine, and their seleno-derivatives. Overall, 17 genes (including sulfate uptake genes) are implicated in the synthesis of adenosylmethionine, methionine and *N*-formylmethionyl-*t*RNA (see Figure 3). Considering that 13 of these genes were upregulated together with the siroheme synthase gene ([cysG-2] NMB1194, siroheme is the cofactor of sulfite reductase), our data unambiguously indicate that sulfur acquisition and metabolism play a key role in the adhesion process of MenB and represent one of the most striking metabolic differences between the two adhering bacteria.

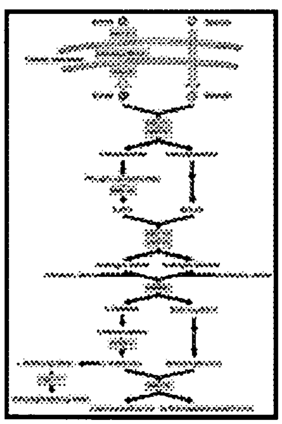

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FIGURE 3. Regulation of genes involved in sulfur acquisition and metabolism. Each arrow represents a specific biochemical step in the overall sulfate and selenate up-take and metabolism of MenB. Genes involved in specific reactions and found upregulated in adhering bacteria are boxed over the corresponding arrows.

Hypothetical Proteins

The most represented gene family responding to cell contact is the family of genes coding for hypothetical proteins (107 genes in MenB, 54 of which are also found in *N. lactamica*). This observation confirms that we still have no knowledge of many genes involved in virulence and cell contact. The list of genes that respond directly to host-cell contact in the two bacteria may provide a useful starting point for further studies. Particularly interesting are the 53 genes specifically induced in *N. meningitidis* since they are likely to play a role in virulence.

Glyceraldehyde 3-Phosphate Dehydrogenase

One of the genes upregulated in both MenB (4.8-fold) and *N. lactamica* (2.7-fold) is gapA-1 (NMB0207), the gene

coding for the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The normal function of GAPDH in cellular metabolism is the conversion of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate with the concomitant production of NADH. However, in some gram-positive bacteria, the enzyme is exported to the bacterial surface. In *Streptococcus pyogenes*, GAPDH represents a major surface exposed protein and acts as an ADP-ribosylating enzyme.[19,20] In *Streptococcus pneumoniae*, the enzyme may be directly involved in the active efflux mechanism of erythromycin.[21] Furthermore, the enzyme plays an important role in cellular communication by activating host protein phosphorylation mechanisms.[22] Finally, in *Staphylococcus*, the cell-surface-associated GAPDH serves as a surface receptor for transferrin and binds different human serum proteins.[23] In MenB, the presence of two GAPDH genes in the chromosome, and the upregulation of one of the two genes following cell contact, suggest a special role for GAPDH. This role was confirmed by FACS analysis, which showed that following cell contact GAPDH is exported to and accumulated on the bacterial surface (data not shown). To our knowledge, this is the first time that GAPDH has been found on the surface of a gram-negative bacterium.

Other Genes

Other genes, belonging to different categories, respond to cell contact and are worth mentioning. For instance, the catalase gene (kat (NMB0216)) was found upregulated in both bacteria. This is consistent with the fact that producing oxygen radicals[24,25] is one of the mechanisms by which eukaryotic cells try to protect themselves against pathogen aggression.

Genes involved in DNA metabolism are often critical for bacterial pathogenesis and, as for DNA restriction-modification genes, are often located within pathogenicity islands[26] or subjected to phase variation.[27-29] In *S. typhimurium*, adenine methylation influences the expression of several virulence genes.[30,31] We found that two restriction modification genes (mod (NMB1261), NMB01375), both encoding DNA methylases and genes coding for nucleases, transposases, helicases, and ligases (NMB0090, recQ (NMB0274), ligA-1 (NMB0666), NMB1251, gcr (NMB1278), and NMB1798) were upregulated during adhesion in both MenB and *N. lactamica*. In addition to these genes, in MenB interaction with epithelial cells promotes transcription of three other DNA metabolism genes (xseB (NMB0262), NMB1510 and mutS (NMB2160)) and three additional transposase genes (NMB1050, NMB1601, NMB1770).

Proteases, chaperonins and proteins involved in protein stabilization, classified as "protein fate" genes, also contribute to the virulence of several pathogens. Our analysis has shown that five genes of this class are upregulated in both *Neisseria* species (prlC (NMB0214), NMB1428, secY (NMB0162), dnaK (NMB0554), hscB (NMB1383)). Eleven "protein fate" genes are MenB-specific and, among these, the only one to be upregulated is the dsbA gene (NMB0278) encoding a periplasmic thiol:disulfide oxidoreductase. In *E. coli*, DsbA plays a role in adhesion by stabilizing type IV fimbriae,[32] and in *Shigella flexneri* it contributes to intracellular survival and propagation.[33]

Recently, Sun *et al.*[10] have developed STM to identify MenB virulence genes. Their study lead to the identification of 73 genes whose inactivation conferred an attenuated phenotype in a mouse model. Nine of the 73 genes were found to be regulated in our analysis, including three genes involved in amino acid synthesis (metF (NMB0943), metH (NMB0944) and gdhA (NMB1710)), the murein transglycosylase B gene (NMB1279), the gene coding for the Cl^- channel protein (NMB2006), the translation elongation factor Tu gene (tufA (NMB0139)), downregulated at 30 minutes of contact with 16HBE14, and three genes of unknown function (NMB0188 and NMB1971, both upregulated, and NMB1523 which was downregulated). Four of these nine genes were MenB-specific (metH, tufA, NMB2006, and NMB1523).

Host-Cell Contact Induces Bacterial Surface Remodeling

Among the adhesion-modulated genes, approximately 44% potentially encode peripherally located proteins suggesting that, when in contact with the host, MenB undergoes a significant remodeling of its membrane components. These biological events have important implications in vaccine design. Particularly abundant antigens or antigens specifically expressed during infection are likely to be better vaccine candidates than proteins whose concentration rapidly decreases when in contact with the host.

In order to confirm that epithelial cell interaction leads to a change in surface protein profile as inferred by microarray data, we used FACS analysis on adhering bacteria using the mouse sera against 12 recombinant proteins whose transcription was found to be particularly activated upon adhesion (see Table 1). All proteins were FACS positive: four of them could be detected on the bacterial surface only after adhesion to epithelial cells, five proteins were present in nonadhering bacteria but their expression increased upon interaction with the host, and three proteins were present on the surface of both adhering and nonadhering bacteria but their expression, differently from their corresponding RNA, did not appear to vary upon epithelial cell interaction.

View this table: **Table 1. MenB proteins selected for FACS and bactericidal analyses**
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Taken together these data confirm that interaction with the host involves substantial modification of surface protein components, and that DNA microarrays coupled to FACS analysis with sera against recombinant proteins is an effective approach to identify surface antigens subject to adhesion-dependent modulation. Interestingly, some of these antigens could not be predicted as membrane-associated by the available computer algorithms. They include glyceraldehyde 3-P dehydrogenase, recently shown to be on the surface of some gram-positive pathogens, *N*-acetylglutamate synthetase and the products of the hypothetical genes NMB1061, NMB1119, and NMB0655.

Identification of Vaccine Candidates

Having identified 12 adhesion-induced antigens localized on the surface of adhering bacteria, we tested the ability of their corresponding antisera to mediate complement-dependent killing of MenB. Of the 12 sera analyzed, 5 showed bactericidal activity against the homologous strain MC58 (Table 1). Two of the bactericidal sera were against hypothetical proteins (the products of NMB0315 and NMB1119 genes) whose function remains to be elucidated. The third bactericidal serum was against the adhesin MafA, one of the two adhesin proteins homologous to gonococcal Maf adhesins.[2] The other two sera were against the MIP-related protein and the enzyme *N*-acetylglutamate synthetase (ArgA).

CONCLUSIONS

Whole-genome microarrays, together with other genomic technologies, were used to study the behavior of each bacterial gene under conditions, which were not accessible to study using previous techniques.[3,4] We have used these techniques to study the changes occurring in gene expression during a critical step of bacterial invasion, that is the first contact between a bacterium and the host cell. In addition, in order to identify the changes that are important for virulence, we have compared

the changes in expression profile between a pathogen (MenB) and a commensal bacteria (*N. lactamica*). The interesting findings discussed in this paper may help to better understand the pathogenicity of MenB and how it differs from the commensal *N. lactamica*. In addition, by following the indication derived from the microarray data, we have investigated the fate of some antigens and confirmed that cell contact indeed promotes a remodeling of the bacterial cell surface antigenic profile. This may prove very useful in rational design of new vaccines. Indeed, 5 out of 12 surface antigens shown to be upregulated after contact to epithelial cells were capable of inducing bactericidal antibodies in the mouse model.

In conclusion, this study not only sheds light on interesting aspects of the first steps of *Neisseria* interaction with its host but also provides a first example of the possible application of DNA microarray technology in vaccine discovery.

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